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Downregulation of cardiac myocyte Na^+-K^+-ATPase by adenovirus-mediated expression of an α-subunit fragment

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Kometiani, Peter, Amir Askari, Jiang Liu, Zijian Xie, and Frederick K. Askari. Downregulation of cardiac myocyte Na^+-K^+-ATPase by adenovirus-mediated expression of an α-subunit fragment. *Am J Physiol Heart Circ Physiol* 280: H1415–H1421, 2001.—Cultured rat cardiac myocytes and A7r5 cells were transfected with an adenoviral vector used earlier for in vivo expression of functional α_2-isoform of the catalytic subunit of rat Na^+-K^+-ATPase. Expressions of truncated forms of α_2, but little or no intact α_2, were detected, suggesting the rapid degradation of α_2 in these cultured cells. In neonatal myocytes normally containing the α_1- and the α_3-isoforms, expression of the α_2-fragment led to *1*) a significant decrease in the level of endogenous α_1-protein and a modest decrease in α_3-protein, *2*) decreases in mRNAs of α_1 and α_3, *3*) decrease in Na^+-K^+-ATPase function measured as ouabain-sensitive Rb^+ uptake, *4*) increase in intracellular Ca^{2+} concentration similar to that induced by ouabain, and *5*) eventual loss of cell viability. These findings indicate that the α_2-fragment downregulates endogenous Na^+-K^+-ATPase most likely by dominant negative interference either with folding and/or assembly of the predominant housekeeping α_1-isoform or with signal transducing function of the enzyme. Demonstration of rise in intracellular Ca^{2+} resulting from α_1-downregulation *1*) does not support the previously suggested special roles of less abundant α_2- and α_3-isoforms in the regulation of cardiac Ca^{2+}, *2*) lends indirect support to proposals that observed decrease in total Na^+-K^+-ATPase of the failing heart may be a mechanism to compensate for impaired cardiac contractility, and *3*) suggests the potential therapeutic utility of dominant negative inhibition of Na^+-K^+-ATPase.

calcium; cardiac glycosides; dominant negative; heart failure; ouabain

OUABAIN AND RELATED CARDIAC glycosides are specific inhibitors of the Na^+-K^+-ATPase that catalyze the coupled active transport of Na^+ and K^+ across the plasma membrane of most higher eukaryotic cells (18, 31). In the heart, cardiac glycosides increase the force of contraction, the positive inotropic effect that is the basis of the continued use of these drugs in the management of congestive heart failure (1, 32, 35). Based on decades of extensive research, the following mechanism for the positive inotropic effect of cardiac glycosides is now widely accepted. The partial inhibition of the cardiac myocyte Na^+-K^+-ATPase that produces a modest increase in intracellular Na^+ concentration ($[Na^+]_i$) is sufficient to affect the sarcolemmal Na^+/Ca^{2+} exchanger to cause significant increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and in the contractile force (1, 32). Interestingly, whereas the reduction of the cardiac Na^+-K^+-ATPase activity by cardiac glycosides is accepted to be responsible for the beneficial effects of these drugs on the failing heart, there is also a large body of evidence (8, 21, 28, 29) to suggest that the development of heart failure, in humans or in experimental animals, is accompanied by reduction in cardiac Na^+-K^+-ATPase. This has led to the suggestion that downregulation of Na^+-K^+-ATPase in the failing heart may be an adaptive response leading to increased contractility by a mechanism similar to that induced by cardiac glycosides (8, 21, 28). It has also been pointed out (28, 29) that the reduced Na^+-K^+-ATPase of the failing heart may exacerbate toxicity of cardiac glycosides in the diseased heart, because the toxic effects of these drugs are known to be the extension of their therapeutic effects. Despite these facts and intriguing speculations, studies on the consequences of the experimentally induced reduction of cardiac Na^+-K^+-ATPase, by means other than drug inhibition, are limited. Valuable information has been obtained from recent studies (16) on the cardiac functions of mice in which the levels of specific isoforms of Na^+-K^+-ATPase were genetically reduced. Using a different approach, here we report studies on cultured rat neonatal cardiac myocytes showing that Na^+-K^+-ATPase function is impaired by the overexpression of a fragment of one of its subunits, and we compare some functional consequences of this downregulation with those of the ouabain-induced inhibition of the enzyme.

EXPERIMENTAL PROCEDURES

Cell preparation and culture. Neonatal rat cardiac myocytes were prepared and cultured as described earlier (15, 26). Myocytes were isolated from ventricles of 1- to 2-day-old Sprague-Dawley rats and purified by centrifugation on Per-

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coll gradients. Cells were plated at a density of $\sim 5 \times 10^4$ cells/cm^2 in a medium containing four parts Dulbecco's modified Eagle's medium and one part Medium 199 (Sigma; St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS). After 24 h of incubation at 37°C in humidified air with 5% CO_2, the medium was changed to one with the same composition, and the cells were used for the indicated experiments. These cultures contained more than 95% myocytes as estimated by immunofluorescence staining with a myosin heavy chain antibody (26). A7r5 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium, 10% FBS, and penicillin-streptomycin as indicated above. After 90–95% confluency, the cells in fresh medium were used for further studies. Calcium-tolerant adult rat cardiac myocytes were prepared as we described earlier (38) and then plated according to Ellingsen et al. (9) except insulin was omitted from the culture medium.

Adenoviral vectors and transfections. The replication-deficient Ad5E1a,E1b,E3b-deleted α_2 (H5.010CMVα_2) and the control β-galactosidase (H5.010CMV*lacZ*) adenovirus-derived expression vectors were made, amplified, and purified as described earlier (5). Cultured cells were washed with the transfection medium, which was the same as the culture medium but with 2% FBS. Cells were then layered with the transfection medium containing the indicated virus titer and rocked intermittently for 90 min. The normal culture medium containing 10% FBS was then added, and cells were incubated at 37°C in humidified air with 5% CO_2 up to 72 h before use as an appropriate assay. Using the control virus and histochemical staining, it was established that at a titer of 10 plaque-forming units (pfu)/cell more than 96% of the cells were infected after 12 h of culture.

Immunoblot analysis. Cultured cells or samples of minced adult rat heart ventricles were washed with ice-cold PBS, collected in 3 ml of a solution containing 0.25 M sucrose, 1 mM EDTA, 30 mM histidine (pH 6.8), 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 50 μg/ml leupeptin, disrupted by sonication, and centrifuged at 100,000 *g* for 30 min at 4°C to obtain a crude membrane preparation. The resuspended pellet was assayed for protein, and equal amounts (usually 65 μg per lane) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were developed using enhanced chemiluminescence and detected by exposure to X-ray film. Images were scanned with a Bio-Rad densitometer to quantitate the relative intensities of the bands. When necessary, multiple exposures of the films or different dilutions of the samples subjected to SDS-PAGE were used to assure that quantitations were made within the linear range of the assay. The primary antibodies used were a monoclonal anti-α_2 (McB2) obtained from Dr. K. J. Sweadner (Massachusetts General Hospital, Boston, MA), a monoclonal anti-α_1 provided by Dr. M. Caplan (Yale University, New Haven, CT), a polyclonal anti-α_2 (residues 335–519), and a polyclonal anti-α_3 (residues 320–514) purchased from Upstate Biotechnology (Lake Placid, NY).

$^{86}Rb^+$ uptake by myocytes. The initial rate of ouabain-sensitive Rb^+ uptake through the Na^+-K^+-ATPase of intact myocytes was assayed by modification of procedures described earlier (26, 37, 38) by using monensin in the assay medium to assure that the maximal capacity of the active uptake was measured (33, 37). Cells cultured and transfected in 12-well plates were washed with fresh culture medium and incubated in the same medium (with its normal Na^+ and K^+ concentrations being 130 and 5.4 mM, respectively) at 37°C for 10 min in the absence or the presence of 1 mM ouabain. Monensin (25 μM) and $^{86}Rb^+$ as the tracer for K^+ (1 μCi/well) were then added to start the uptake experiment. After 20 min, 3 ml of ice-cold 100 mM $MgCl_2$ were added to stop uptake. Cells were then washed in the same solution, dissolved in SDS, assayed for protein, and counted by conventional procedures. It was established in preliminary experiments that uptake was a linear function of time for the duration used.

Fluorescence microscopic assay of $[Ca^{2+}]_i$. Fluorescence microscopic assay of $[Ca^{2+}]_i$ was done using fura 2 (Molecular Probes, Eugene, OR) as previously described (26, 37). Myocytes were loaded with 5 μM fura 2-acetoxymethyl ester for 30 min at room temperature and perfused with the normal medium or the medium containing ouabain for 15 min before measurements were made on 20 different cells. Single cell fura 2 fluorescence was recorded using an Attofluor imaging system (Atto Instruments, Rockville, MD) at excitation wavelengths of 340–380 nm and an emission wavelength of 505 nm. Measurement of time-averaged signals on each cell was completed in 30 s. Calibration procedures and calculations described earlier (26) were used to relate the fluorescence ratio (340:380) to $[Ca^{2+}]$.

Other assays. Northern blots were done as previously reported (5, 13, 26) using glyceraldehyde-3-phosphate dehydrogenase mRNA for normalization and quantitation of the blots. Viability assays were conducted as in our earlier study (26) by the measurements of total and released lactic dehydrogenase using a kit (Sigma). Protein was determined by the Bio-Rad DC protein colorimetric assay (Bio-Rad, Hercules, CA).

Analysis of data. Data are means ± SE of the results of a minimum of three experiments. Student's *t*-test was used, and significance was accepted at $P < 0.05$. The presented Northern and Western blots are representative of the results of the multiple experiments.

RESULTS

Expression of truncated α_2-subunit in rat cardiac myocytes and A7r5 cells. It is known that the freshly cultured neonatal rat cardiac myocytes express the α_1- and the α_3-isoforms of the catalytic subunits of Na^+-K^+-ATPase, but not the α_2-isoform (3, 19, 24). In relation to our studies on the role of Na^+-K^+-ATPase in the regulation of the growth of these neonatal cells (13, 14, 17, 26), we were interested in learning whether the α_2-subunit of the rat enzyme could be overexpressed in these cells by transient transfection. Because in previous studies we used an α_2-adenoviral vector for the in vivo expression of the functional α_2-protein in rat liver (5), we used the same vector to transfect the neonatal myocytes, prepared cell lysates at various times after transfection, and subjected these to SDS-PAGE and immunoblotting using a monoclonal anti-α_2-antibody (McB2). As shown in Fig. 1, the intact α_2 with the apparent relative molecular mass of ~100 kDa, which is known to be present in the myocytes of the adult rat heart (3, 6, 21, 22), was not detected in these neonatal cells, but there was significant time-dependent expression of an immunoreactive band with the apparent relative molecular mass of ~60 kDa. The expression of this band rose significantly up to 24 h after transfection (Fig. 1), but remained constant thereafter up to 72 h (not shown). Its expression was also affected by

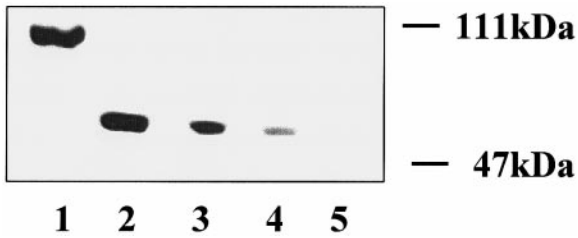

Fig. 1. Time-dependent expression of a truncated α_2-subunit in neonatal cardiac myocytes transfected with the adenoviral α_2-vector. Cultured myocytes were transfected [20 plaque-forming units (pfu)/cell] with the α_2- or the control virus as described in EXPERIMENTAL PROCEDURES. At indicated times after transfection, crude membrane preparations were made, and equal amounts were subjected to SDS-PAGE and then probed with an anti-α_2-antibody. *Lane 1*, positive control, adult rat heart; *lanes 5*, *4*, *3*, and *2*, myocytes transfected with the α_2-virus after 6, 12, 18, and 24 h, respectively. Untransfected control and those transfected with the control virus were similar to *lane 5* (not shown).

the virus-to-cell ratio during transfection reaching a maximum at 20 pfu/cell and then declining (Fig. 2). Because the epitope for McB2 is close to the NH_2-terminus of the α_2 (25) and because the expressed 60-kDa protein was also detectable with a polyclonal antibody against residues 335–519 of the α_2 (data not shown), the expressed 60-kDa protein seems to be the α_2-subunit that is truncated within the large central cytoplasmic loop containing the ATP binding sites and the site phosphorylated during ATP hydrolysis (18).

To see whether the expression of the truncated α_2 was a peculiarity of the neonatal myocytes, cultured adult rat cardiac myocytes and A7r5 cells, a rat smooth muscle cell line, were transfected with the α_2-vector and subjected to Western blot analysis. In adult myocytes, limited experiments similar to those of Figs. 1 and 2 did not reveal significant changes in the low basal level of intact α_2, but showed significant expression of the same 60-kDa band shown in Fig. 1 (data not

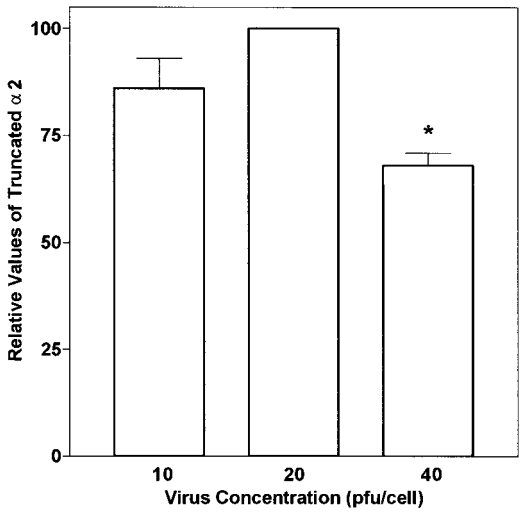

Fig. 2. Relative amounts of truncated α_2 expressed 48 h after transfection as a function of virus titer. Experiments were done as described in Fig. 1 and EXPERIMENTAL PROCEDURES. Each value is expressed as percentage of the maximal value obtained. *$P < 0.05$; $n = 5$.

Fig. 3. Detection of immunoreactive α_2-subunit and its fragments in A7r5 cells transfected with the α_2-adenovirus. A7r5 cells were transfected and immunoblotted as indicated in Fig. 1. *Lane 1*, control, adult rat heart; *lane 5*, untransfected A7r5 cells; *lane 4*, cells transfected with the α_2-virus after 12 h, *lane 3*, 24 h; and *lane 2*, 48 h.

shown). Of particular interest were the findings on A7r5 cells. As shown in Fig. 3, transfection with the α_2-vector resulted in a modest time-dependent increase in the level of intact α_2 and in significant expression of two α_2-fragments of ~60 kDa and 34 kDa. The Northern blot analysis of total cellular RNA from neonatal myocytes and A7r5 cells transfected with the α_2-virus showed the presence of the appropriate α_2-specific message of the same size as that we showed earlier in the transfected rat livers (5). Although the detailed mechanisms involving the expression of the truncated α_2-fragments in cardiac myocytes and A7r5 cells remain to be determined (see DISCUSSION), the results of the above experiments on myocytes and A7r5 cells suggest that the expression of the intact α_2-subunit in the transfected cells is accompanied by its rapid degradation, with the most stable product being a 60-kDa fragment. In the remaining experiments described below, we address the issue of the consequences of the overexpression of the truncated α_2 in the neonatal myocytes.

Downregulation of α_1- and α_3-subunits in neonatal myocytes. The accumulation of the truncated α_2 raised the question of whether this interfered with the expressions and/or functions of the endogenous α-subunits. To determine the effects on the levels of the endogenous α_1- and α_3-subunits, neonatal myocytes were transfected with α_2 or a control virus at different virus-to-cell ratios, and changes in α_1- and α_3-proteins were quantitated after 2 and 3 days using isoform-specific antibodies. The level of α_3-protein was not changed significantly 2 days after transfection (not shown), but was modestly reduced after 3 days (Fig. 4); whereas the α_1-protein was significantly downregulated in a dose- and time-dependent manner (Fig. 4). The differential reductions of the α_1- and the α_3-protein levels point to the specificity of the effects of transfection with the α_2-virus.

To determine whether the α_1- and α_3-mRNAs were also affected by the expression of the truncated α_2, myocytes were transfected with either the α_2-virus or the control virus, at 20 pfu/cell for 48 h as in Fig. 4, and subjected to Northern blot analysis. Both α_1- and α_3-mRNAs were significantly reduced in the α_2-transfected cells relative to those transfected with the control virus (Fig. 5), suggesting that this may be responsible, at least in part, for the reduced α_1- and α_3-protein levels noted in Fig. 4.

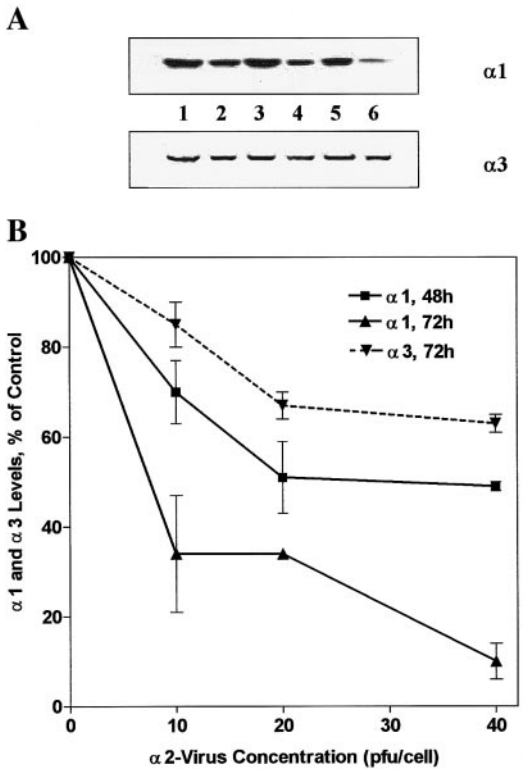

Fig. 4. Downregulations of the α_1- and the α_3-subunit proteins in neonatal cardiac myocytes transfected with the α_2-adenovirus. Myocytes were transfected with the α_2- or the control virus at the indicated titers and cell membrane preparations were subjected to SDS-PAGE and assayed for intact immunoreactive α_1- and α_3-subunits, with isoform-specific antibodies after 48 and 72 h as described in EXPERIMENTAL PROCEDURES. The subunit levels in cells transfected with the control virus did not differ significantly from those in untransfected cells. *A*: representative blots for α_1- and α_3-subunits. *Lanes 1*, *3*, and 5 were transfected with the control virus at 10, 20, and 40 pfu/cell, respectively. *Lanes 2*, *4*, and *6* were transfected with the α_2-virus at 10, 20, and 40 pfu/cell, respectively. *B*: quantitative comparisons of multiple blots, such as those shown in *A*. For the α_2-transfected cells, each value is expressed relative to the corresponding value for the cells transfected with the control virus. Standard errors not shown when smaller than the symbol size. For the 72-h points, $n = 3$; for the 48-h points, $n = 6$.

Functional consequences of the expression of the truncated α_2 in neonatal myocytes. The following experiments assessed the functional consequences of the transfection of the neonatal myocytes with the α_2-virus and the resulting downregulation of the endogenous α-subunits. We deemed it essential to assay the ion transport capability of the enzyme in intact cells rather than ATPase activity or a partial reaction of the enzyme in broken cells to assure that the assembled functional enzymes of the plasma membrane were being measured.

Experiments shown in Fig. 6 showed that ouabain-sensitive Rb^+ uptake, an established measure of the transport function of Na^+-K^+-ATPase, was reduced significantly as a function of increasing virus-to-cell ratio. Because in these neonatal myocytes the α_1-subunit constitutes ~70–80% of the total α-content (19, 34, 39), the data in Figs. 4 and 6 indicate that the reduced transport function of the transfected cells must be predominantly, if not entirely, due to the downregulation of the α_1-subunit.

In cardiac myocytes, the most prominent intracellular ionic change resulting from the partial inhibition of Na^+-K^+-ATPase by cardiac glycosides is the increase in $[Ca^{2+}]_i$ (1, 32). Experiments in Fig. 7 show $[Ca^{2+}]_i$ was indeed increased in myocytes transfected with the α_2-virus but not in those transfected with the control virus. The transfection protocol used in experiments shown in Fig. 7 was expected to cause ~40–60% reduction in the transport function of Na^+-K^+-ATPase (Fig. 6). For comparison, the effects of 0.1 mM ouabain, which is also known to cause ~50% inhibition of Na^+-K^+-ATPase of these myocytes (26, 39), were also determined on $[Ca^{2+}]_i$. As expected, exposure to ouabain caused significant increases in the control cells and a further increase in the α_2-transfected myocytes (Fig. 7).

High levels of ouabain-induced inhibition of Na^+-K^+-ATPase in cardiac myocytes leads to Ca^{2+} overload and loss of viability (26). Experiments were done, therefore, to compare the viabilities of untransfected cells and those transfected with the control virus or the α_2-virus. There were no significant differences between the groups after 48 h of culture (data not shown). After 72 h, transfection with the α_2-virus, but not the control virus, led to dose-dependent increase in the loss of

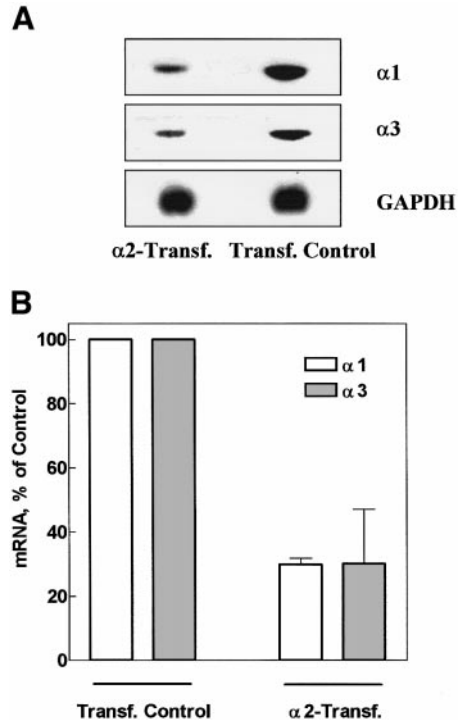

Fig. 5. Downregulations of the α_1- and α_3-subunit mRNAs in neonatal cardiac myocytes transfected with the α_2-adenovirus. Myocytes were transfected with α_2- or the control virus at 20 pfu/cell for 48 h and subjected to Northern blot analysis using the appropriate probes for α_1- and α_3-subunits, as indicated in EXPERIMENTAL PROCEDURES. *A*: representative autoradiogram. *B*: quantitative comparisons of the α_1- and the α_3-mRNAs, normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. For each isoform, the value in the α_2-transfected cells is expressed relative to that in the transfected control cells ($n = 3$).

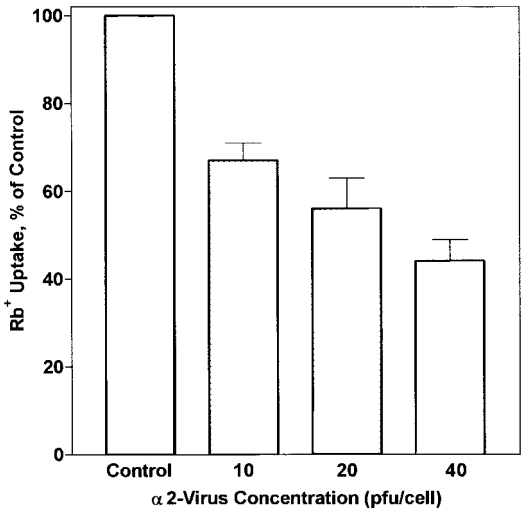

Fig. 6. Reduction of ouabain-sensitive $^{86}Rb^+$ uptake in neonatal myocytes transfected with the α_2-adenoviral vector. Myocytes were transfected with the α_2- or the control virus at the indicated titers and after 48 h were assayed with untransfected controls for the initial rate of ouabain-sensitive Rb^+ uptake as indicated in EXPERIMENTAL PROCEDURES. The results did not differ significantly in untransfected cells and those transfected with the control virus. For the α_2-transfected cells, each value is expressed relative to the corresponding value for the cells transfected with the control virus ($n = 3$).

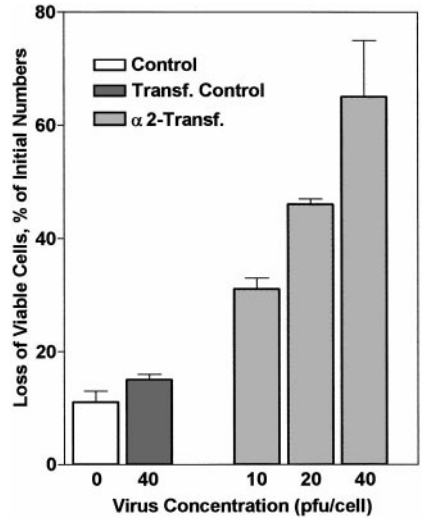

Fig. 8. Effect of the transfection with the α_2-adenoviral vector on the viability of the neonatal myocytes. Myocytes were transfected with the indicated titers of the α_2- or the control virus. After 72 h, loss of viable cells in the untransfected cells and the transfected cells was estimated as indicated in EXPERIMENTAL PROCEDURES ($n = 3$).

viable cells (Fig. 8) clearly indicating the expected eventual consequence of the downregulation of the housekeeping Na$^+$-K$^+$-ATPase in cardiac myocytes.

DISCUSSION

This study was initiated with the original aim of assessing the consequences of the expression of the functional α_2-subunit of the rat Na$^+$-K$^+$-ATPase in the neonatal rat cardiac myocytes lacking this isoform. It soon became evident, however, that only a truncated α_2-subunit not likely to be functional was overexpressed in these cells. Because enzyme and receptor fragments may often act like inactive mutant variants and cause dominant negative inhibition (2, 10, 12, 23, 27, 36) we attempted to determine whether the expression of the α_2-fragment impaired the function of endogenous Na$^+$-K$^+$-ATPase in the neonatal myocytes. Our findings clearly show that the ion transport function of Na$^+$-K$^+$-ATPase is indeed inhibited concomitant with the expression of the truncated α_2-isoform and that this is accompanied by a significant reduction of the α_1-protein content of the neonatal myocyte. Because the induced reduction of the α_3-protein content is small, if any, and because it is established that α_1 constitutes ~70–80% of the total α-content of these myocytes (19, 34, 39), it is clear that the induced downregulation of α_1-protein is responsible for the reduced transport function associated with the expression of the α_2-fragment in the transfected cells.

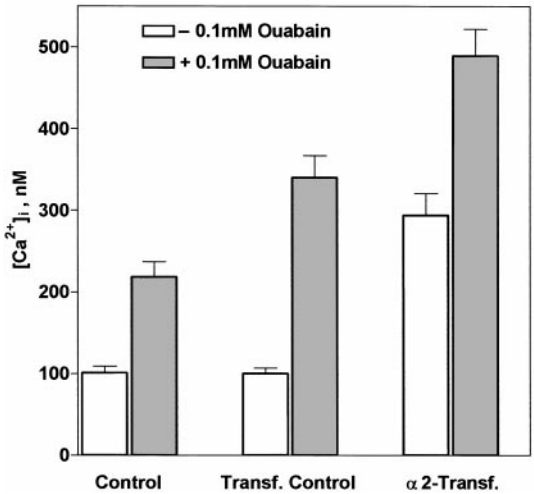

Fig. 7. Comparison of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in control and transfected neonatal myocytes in the absence and the presence of 0.1 mM ouabain. Myocytes were transfected (20 pfu/cell) for 48 h with the α_2- or the control virus. These and untransfected controls were washed, loaded with fura 2, and assayed for $[Ca^{2+}]_i$, as indicated in EXPERIMENTAL PROCEDURES, before and after exposure to ouabain. Each value is the mean ± SE of determinations in 20 different single cells.

Although the noted changes in the α_1- and α_3-mRNAs that accompany the expression of the α_2-fragment are sufficient to account for reductions in α_1- and α_3-proteins, the possibility that translation or degradation of these subunits is also affected cannot be ruled out. Regardless of whether it is the message, or the protein turnover, or both that are altered, the question arises as to how the expression of the α_2-fragment may affect these processes. The overexpression of a fragment of a protein, or an inactive mutant, may disrupt the function of the wild-type gene product either by interference with the proper folding of the monomeric protein, or by blocking of its assembly into a functional oligomer, or by preventing its transient functional interaction with another protein or DNA (12, 23). For the transport function of Na$^+$-K$^+$-ATPase, assembly of the α-subunit with the β-subunit is obligatory (18, 31), and

α,α-interactions are also necessary for normal function, at least within the native membrane (4). The overexpression of the α_2-fragment, therefore, may affect the folding of the endogenous α-subunits or their assembly into the functional oligomers, either of which would result in enhanced degradation and downregulation. Recent studies (11, 17, 26, 37) indicate that Na^+-K^+-ATPase also functions as a signal transducer, regulating a number of transcription factors through pathways beginning at the plasma membrane with stimulus-induced protein-protein interactions involving Na^+-K^+-ATPase, Src, the epidermal growth factor, and adaptor proteins. Therefore, by disruption of such protein-protein interactions, a fragment or a mutant of a Na^+-K^+-ATPase subunit may also exert transcriptional effects. Which one of the above possible mechanisms accounts for the dominant negative inhibition by the α_2-fragment remains to be determined.

Perhaps the most significant aspect of the present study is the finding that the downregulation of the α_1-isoform and the associated reduction of the transport function of Na^+-K^+-ATPase lead to an increase in $[Ca^{2+}]_i$ comparable to that induced by the partial inhibition of Na^+-K^+-ATPase with ouabain. To our knowledge, this is the first demonstration that the reduction of functional cardiac Na^+-K^+-ATPase by means other than drug-induced inhibition causes an increase in $[Ca^{2+}]_i$. In this regard, it is important to note that in recent elegant studies (16) on the cardiac function of mice in which either the α_1- or the α_2-subunit level was genetically reduced, no changes in the resting $[Ca^{2+}]_i$ of the myocytes were noted, and it was not possible to measure the ion transport function of the myocyte Na^+-K^+-ATPase. Several implications of our findings concerning the association of rise of $[Ca^{2+}]_i$ with the downregulation of α_1 are worthy of note. First, this provides some measure of indirect support for the repeated conjecture that downregulation of Na^+-K^+-ATPase associated with heart failure may indeed be an adaptive response resulting in rise of $[Ca^{2+}]_i$ and improvement of the impaired contractility (8, 21, 28, 29). Second, that the increase in $[Ca^{2+}]_i$ is primarily due to the downregulation of the predominant α_1-subunit adds to the weight of evidence against the hypothesis that the less abundant α-isoforms (α_2 or α_3) of the cardiac myocytes have a special role in the regulation of $[Ca^{2+}]_i$ because they are colocalized along with the Na^+/Ca^{2+} exchanger in the transverse tubules or other specialized plasma membrane domains (7, 16, 20). While this hypothesis may be consistent with some observations (7, 16), it is not supported by studies on the subcellular distributions of the isoforms in mammalian cardiac myocytes (22). The present findings certainly do not rule out special roles for α_2- and α_3-isoforms. In agreement with others (8, 22), however, our findings are more in accord with the proposal that either the downregulation or the cardiac glycoside-induced inhibition of any isoform of cardiac Na^+-K^+-ATPase contributes to the elevation of $[Ca^{2+}]_i$ and increased contractility, as long as the isoform is capable of active transport of Na^+ and K^+ across the plasma membrane.

Finally, although the determination of the cause of the expression of the truncated α_2 is not the focus of this report, it is appropriate that we briefly address this issue. The adenoviral α_2-vector used here produces a functional full-length α_2-subunit in the intact rat liver (5). If the α_2-fragments noted in the transfected myocytes and A7r5 cells are indeed due to the initial expression of the intact α_2 and its subsequent fragmentation as suggested by our data, the question arises as to why the expressed full-length α_2 is less stable in the cultured cells used here than in the intact liver. An obvious possibility is the cell specificity of the turnover process. An alternative is that the α_2-truncation represents a degradation process different in the intact organ than in the cultured cell. This has some indirect experimental support. Recent studies (30) have shown that when the neonatal rat skeletal muscle containing significant levels of α_1- and α_2-subunit proteins is dispersed and cultured, no α_2 is detected after a day in culture, whereas the level of α_1 is not decreased. Also pertinent are the hormonal and neurogenic control of the α_2 turnover (3, 20, 24) that clearly must be different in cultured and in vivo cells. The mechanisms involved in the expression of truncated α_2 under the conditions used in our studies and their possible relation to the in vivo regulation of the α_2 turnover remain to be explored. Regardless of how these issues are resolved, the present study clearly suggests that cardiac $[Ca^{2+}]_i$ may be regulated by the expression of Na^+-K^+-ATPase subunit fragments and points to the potential therapeutic use of the dominant negative impairment of Na^+-K^+-ATPase function as an alternative to its drug-induced inhibition.

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